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A New Laser Pointer Driven Optical Microheater for Precise Local Heat Shock

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**A NEW LASER POINTER DRIVEN OPTICAL MICROHEATER FOR PRECISE
LOCAL HEAT SHOCK**

A Thesis Presented

by

MIKE PLACINTA

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2009

Molecular and Cellular Biology Program

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DEDICATION

Dedicated to my mother (Maria Placinta) and my father (Simion Placinta)

ACKNOWLEDGMENTS

I would like to express my deepest and sincere gratitude to my supervisor Rolf O. Karlstrom as my patient mentor and supporter in this project. This research would not be possible without Rolf's support, ideas, assistance, and encouragement.

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ABSTRACT

A LASER POINTER DRIVEN OPTICAL MICROHEATER FOR PRECISE LOCAL HEAT SHOCK

SEPTEMBER 2009

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The zebrafish has emerged as an important genetic model system for the study of vertebrate development. However, while genetics is a powerful tool for the study of early gene functions, the approach is more limited when it comes to understanding later functions of genes that have essential roles in early embryogenesis. There is thus a need to manipulate gene expression at different times, and ideally only in some regions of the developing embryo. Methods for conditional gene regulation have been established in *Drosophila*, *C.elegans* and the mouse, utilizing conditional gene activation systems such as the Gal4-UAS system (fly) and the cre/lox recombination system (mouse). While these tools are also being developed in zebrafish, the accessibility of the zebrafish embryo makes other approaches both possible and desirable.

We have taken advantage of a heat-shock inducible system that uses the *hsp70* promoter that is activated by cellular stress, such as heat. Having established that this global heat shock method allows temporal control of gene expression, we aimed to spatially control gene expression by applying controlled thermal heat to only a small region of the embryo. This would allow us to determine cell- and tissue-autonomous

roles for developmentally important genes in an embryo with otherwise normal gene function. We have now developed a device that uses a laser to heat a defined region of the embryo, and thus activate the *hsp70* promoter only in restricted regions of the embryo. The output of a 75 mW red laser pointer was focused into the 50 μ m diameter core of an optical fiber, whose cleaved and coated end was used to heat, and thus induce, gene expression in a defined area. We have established conditions that allow controlled heating and trans-gene activation in small regions of the embryo without inducing cell death. This new tool will allow us to study the cell-autonomous roles of embryonic signaling molecules in cell differentiation, proliferation, and survival in a variety of tissues and at different times.

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

Over the last few decades considerable insight has been gained into the genes that control normal embryonic development. Much of this has been possible through the use of methods that perturb the normal expression of a gene of interest. Most commonly, this involves modifying *where*, *when* and at what *level* the gene is expressed.

Gain of function and loss of function analyses are major tools for uncovering how gene products control development (Key and Devine, 2003). Elimination of gene function through genetic knockout or anti-sense approaches can illuminate the earliest role a gene plays in development, uncovering the processes for which a gene is necessary. Similarly, over-expression experiments allow researchers to determine what developmental events a gene is sufficient to regulate. While these global gene loss and gain of function approaches are extremely informative, they are more limited when a gene plays multiple roles throughout development. This is particularly troublesome when a gene is essential for some early embryonic process, since later processes can then not be studied.

Use of the Cre-Lox system to control gene expression

On this basis, much effort has gone into developing methods to perturb gene expression in a temporally, and/or spatially, restricted manner. In mice, and to a lesser extent, zebrafish, the Cre-Lox recombination system, that enables site specific recombination of a target DNA sequence, has been used as a tool for genetic manipulation (Fig.1-1). In this system, Cre is a key enzyme that catalyzes the

recombination of DNA between specific sites (lox sites). Specific target genes can be engineered to include flanking Lox sites, facilitating their excision in the presence of Cre recombinase. This Cre-Lox system has been used in different model organisms to alter gene expression in all cells, specific tissues, or subsets of cells (eg. nerve cell, mammalian cells, heart cells and etc.).

This system has been used extensively in creating transgenic mice (Kuhn and Torres, 2002), and can allow temporal control of gene expression by having it under the control of an inducible promoter such as *hsp70*. Potentially, the Cre-Lox system may be adapted for clinical purposes, for example in the control of cancer genes (Bex et al., 2002). Moreover it has been shown to be effective when it is linked to a heat shock promoter to facilitate the study of oncogenes in the zebrafish embryo (Le et al., 2007).

The efficiency of the Gal4-UAS system in different model systems

A different way to study and analyze gene function in different tissues is to use the Gal4-UAS system. The Gal4 protein is known to be a transcriptional activator that binds to DNA sequences located in upstream regulatory sites called the UAS (upstream activation sequence). Gene expression is controlled by expressing Gal4 only in some cells or at some times in animals carrying a UAS-transgene. Gal4 expression can be controlled by tissue specific gene regulatory elements (Fig.1-2). This Gal4-UAS construct enables researchers to control and/or direct specific genes for transcription in *Drosophila* (Fischer et al., 1988). The Gal4-UAS can be also linked to a reporter gene such as EGFP to study and compare different amounts of gene expression during developmental stages. This Gal4-UAS system is being used widely in fly and is still

being developed in zebrafish (Asakawa and Kawakami, 2008; Asakawa et al., 2008).

This system has also been applied to *Xenopus* to study molecular mechanisms underlying embryogenesis, highlighting the potential for the Gal4-UAS system as a tool for the study of gene expression (Hartley et al., 2001).

The use of antisense technology in studies of early embryonic development

When it comes to knocking down the function of a gene of interest, antisense technology has become an important tool. For example, in zebrafish and *Xenopus*, morpholinos have been widely used to study gene function. Morpholinos are DNA analogs which knock down the expression of target genes, most commonly by sterically hindering ribosome binding and preventing translation of the protein (Heasman, 2002). RNA interference (RNAi), for example is now used for therapeutic purposes in silencing viruses that have the RNA construct and thus could possibly be used in currying many diseases in the near future. RNAi plays an important role in regulating gene expression by selectively turning off synthesis of target genes through triggering the destruction of mRNA or silencing the transcription of a gene. Thus, the difference between RNAi and morpholinos lies in the mechanism they use to knock down gene function. Despite the successful application of RNAi in *C.elegans* and mouse, it has been reported that RNAi has some non-specific and deleterious effect on zebrafish embryos (Zhao et al., 2001). Although morpholinos work well in zebrafish, their potential is limited to knock down gene function at later developmental stages. This mostly due to problems in delivery of the morpholino at later stages, however it has also been noted that the concentration of the morpholino in each cell decreases progressively as the animal grows, and so the

morpholino concentration reaches a level where it is below threshold to block gene function.

All of the tools or methods described above allow spatial and/or temporal regulation of genes, but their utility becomes more limited when we want to perturb target gene expression in a spatially restricted region and at a specific time during development. Some of these tools have yet to be optimized for use in the zebrafish embryos (Robu et al., 2007), limiting our ability to study some critical aspects of an organism's early development. For example, the identification of genetic mutants which encode components of the Hh signaling pathway have provided great insight into the roles of this signaling pathway during embryogenesis, however are of no use to understanding later roles of Hh signaling in cancer and adult stem cell populations maintenance due to their early mortality. Similarly, morpholinos are a powerful tool for the study of the early roles of hedgehog in mutants (Bingham et al., 2001) but become more difficult to apply to later gene function, necessitating electroporation or other means of introducing morpholinos at older embryonic and adult stages (Cerdeira et al., 2006).

Heat shock promoters: an alternative tool to analyze developmental genes

An alternative method for the spatial and temporal regulation of gene function is to take advantage of a well described heat shock promoter, the *hsp70* promoter. The *hsp70* gene is found in many organisms including mice, humans and zebrafish. The normal role of this heat shock protein is to protect cells in different organs and/tissues from stress such as heat, viral infection, toxic stress or other detrimental agents that increase cellular stress (Whitley et al., 1999). The *hsp70* protein normally acts as a

chaperone, ensuring that proteins that are important in cells undergo proper folding following heat stress. Because transcription of *hsp70* is rapidly upregulated following cellular stress, the *hsp70* promoter can be utilized as an inducible promoter to alter gene expression during development.

The *hsp70* promoter has been used in zebrafish to activate transgene expression throughout the embryo (Shoji and Sato-Maeda, 2008b). For example, the heat shock promoter has given insight into gene function that would not have otherwise been possible in examining the roles of Bmp and Fgf signaling in early liver development (Shin et al., 2007), and also determining the role Wnt signaling in neural crest induction in the zebrafish embryo (Lewis et al., 2004). The availability of *hsp70* driven transgenes opens the door to temporal regulation if the tissue could be locally heated. A precise heat shock device that would allow the activation of transgene expression at any stage of development in specific tissues and/or a subset of cells.

Established methods for the temporal regulation of genes using *hsp70*

It has been shown that global heat shock turns ON transgene expression (e.g. *hsp-GFP*) throughout the entire embryo (Shoji and Sato-Maeda, 2008a). Laser local heat shock in zebrafish was first reported by Halloran et al., (2000), where they used a Micropoint laser focused through a compound microscope to induce expression of a fluorescent reporter gene in single muscle cells in transgenic zebrafish embryos [*Tg(hsp70:GFP)*] (Fig. 1-3 A, B). While this method of temporal gene induction is effective to activate gene expression in single cells, its utility is limited by expense and questions of cell viability have been raised. Ramos et al., (2006) used a powerful 532 nm

continuous wave laser that projected the laser beam into positioning mirrors to locally induce gene expression in *Drosophila*. However, the projection of the beam through positioning mirrors does not give precise regulation of cell death, which is a problem with this method. A more recent study has shown that infrared laser microscopy (IR-LEGO) can be used to locally activate single cells in transgenic *C. elegans* under the control of a heat shock promoter. The infrared light offers higher wavelengths than the Micropoint laser used by Halloran et al., (2000), with a higher efficiency of activating desired cells.

Recently, Hardy et al demonstrated that it was possible to locally induce gene expression in transgenic live zebrafish embryos using a soldering iron device (Hardy et al., 2007). This device, while seemingly effective, requires a reservoir for cooling the medium, and can be used only for superficial cells. In addition, there is no means by which to precisely control the temperature of the tip of the soldering iron device, which has implications for the reproducibility of the experimental conditions.

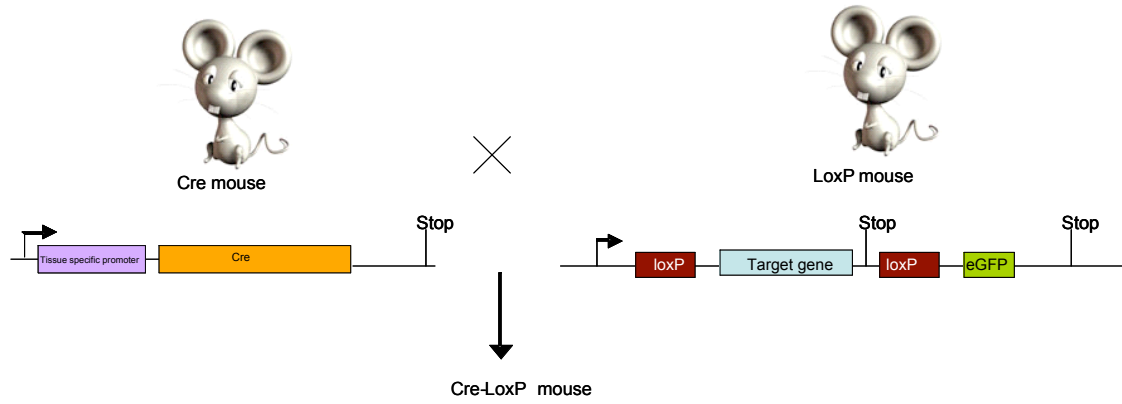
Spatial and temporal gene induction using a new optical microheater

On this basis, my aim was to develop a simple local heat shock device that would allow us to consistently and reproducibly induce gene expression in zebrafish embryos and larvae, without the requirement for a cooling system, and without causing cell death. We first developed a small electrically controlled wire heater that contacts a small area of the embryo in order to activate gene expression in a defined region. We used an Omega Thermo-Controller device to monitor and control electric current to small igniter wires. However, this system was extremely hard to control since the wire at the tip of the

heating probe constantly moved with the pulsing current from the Omega controller and the temperature of the tip itself was unstable.

We then decided to take a different approach that employed a laser pointer with enough power to heat the end of a fiber optic tip. 20-150 μm scale fiber optics could be placed next to embryonic tissue to locally raise the temperature and activate the *hsp70* promoter. Our new laser tool is an alternative to the costly Micropoint laser, and is more precise and easier to use than other laser-based systems. The energy from a 75 mW laser pointer is absorbed by ink and converted into heat, therefore allowing us to precisely regulate gene expression in a number of cells without causing tissue damage. This device is easy to control by varying the current to the laser. Since the heat is restricted to the fiber optic tip, no thermal cooling is needed. The device is low-cost and quick to set up. We have now extensively tested this device on zebrafish embryos and larvae. After optimization, our success rate for activating transgene expression is close to 100%. Importantly, transgene activation occurs in the absence of noticeable tissue damage and cell death. Our experiments reveal that local heat shock with a 650 nm optical laser is precise, simple and fast enabling the study of gene function at any time in the life cycle of the zebrafish. In addition, it has clear application for fate mapping studies.

F0 Generation



F1 Generation

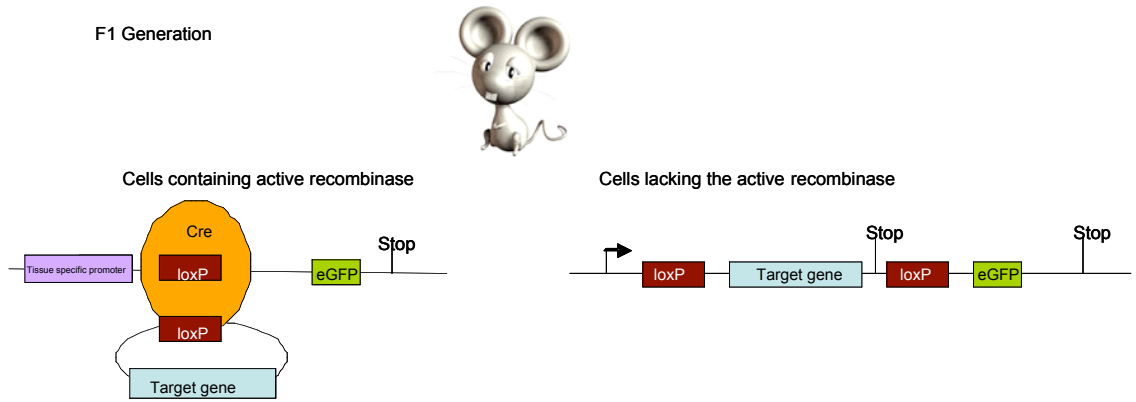
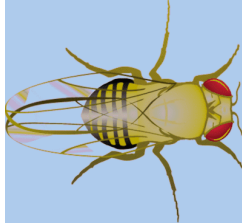


Figure 1-1. Overview of Cre/Lox system for conditional gene regulation.

The Cre recombinase enzyme binds to lox sites on the transgenic construct causing excision of the region between the two lox sites and expression of the reporter gene (GFP in this case). By using a tissue specific promoter, cre is expressed only in certain tissues, resulting in loss of gene function only in these cells. Alternatively, other conditional promoters can be used to activate the cre transgene. In the F1 generation the original gene function is distorted, allowing a reporter gene (eg. eGFP) to be transcribed instead. (Adapted from Sauer, 1998).

Enhancer Trap GAL4



UAS-Gene X

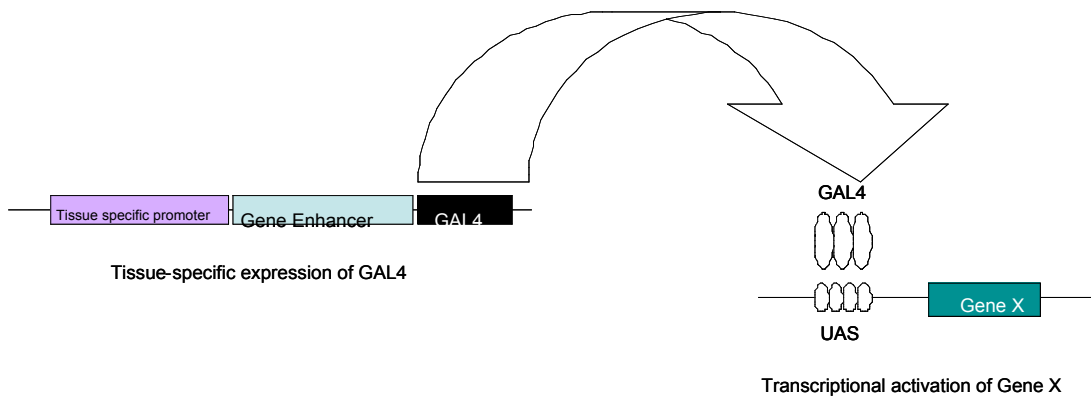
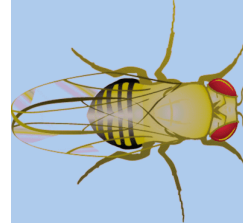


Figure 1-2. Overview of Gal4-UAS system for conditional gene regulation.

Gal4 is a transcription factor that is used to drive conditional gene activation when placed under the control of a specific promoter. The Gal4 activator line is crossed to another line that carries the UAS sequence upstream of gene X (target gene). In the offspring of this cross, Gal4 will bind the UAS and activate transcription of the downstream target gene. The context for this target gene activation will be determined by the promoter used to drive Gal4 expression. (Adapted from Brand and Perrimon, 1993).

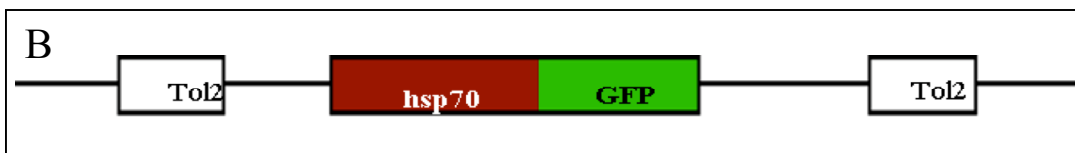
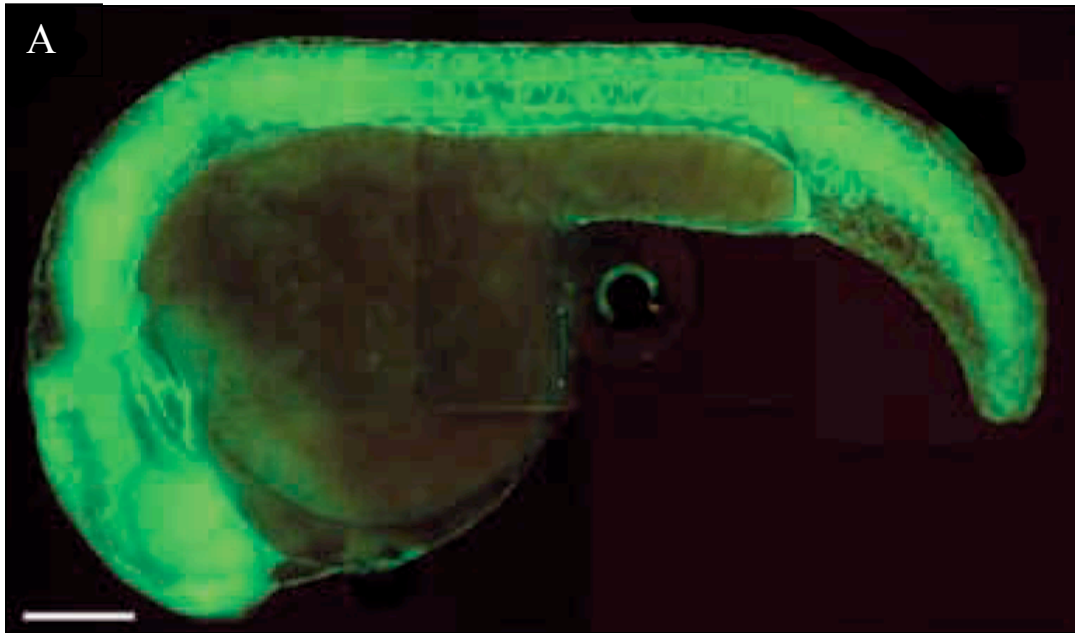


Figure 1-3. The heat shock promoter is activated by increasing the thermal energy.

- A) A transgenic zebrafish line carrying the hsp70-GFP transgene was expressing GFP in all every cell in the embryo, Mary Halloran (Halloran et al., 2000). Scale bar: 10 μ m.
- B) Zebrafish *hsp70* transgene constructs used to generate transgenic lines used in this study: *hsp70:GFP*.

CHAPTER II

A LASER POINTER DRIVEN MICROHEATER FOR PRECISE LOCAL HEATING AND CONDITIONAL GENE REGULATION IN VIVO

Abstract

The study of gene function is greatly facilitated by technologies that allow conditional regulation of gene expression. The optical accessibility of the zebrafish embryo prompted us to develop a laser based optical method for spatially and temporally controlling transgene expression. Here we describe a simple micro-heater that employs a micron-scale fiber optic and a laser-pointer as a power source. Optical fibers can be pulled on a standard electrode puller to produce tips of varying sizes that can then be used to heat small regions of the zebrafish embryo. We demonstrate reliable transgene activation in 20-100 μm regions of zebrafish embryos and larvae. This versatile and simple local heater has broad utility for the study of gene function and for lineage tracing throughout the life cycle. In addition, this device could also be adapted for therapeutic purposes in humans.

Introduction

The study of gene function has been greatly facilitated by the ability to conditionally regulate gene expression at different times and locations throughout the life cycle. A variety of genetic tools that allow such temporal and/or spatial control of gene expression have been developed for study of gene function in a variety of model organisms. These include site-specific recombination using cre or flp recombinases (Bischof and Basler, 2008; Feil, 2007) (Hans et al., 2009), tetracycline inducible systems (Huang et al., 2005), and the Gal4/UAS system (Halpern et al., 2008; Lewandoski, 2001). Another system for conditional gene regulation takes advantage of the cellular heat shock response, which leads to the transcription of a variety of genes that allow cells to tolerate brief periods of stress, or to activate cell death pathways when these stresses are too extreme (Arya et al., 2007; Lindquist, 1986). One particularly well studied heat shock inducible gene is the *hsp70* gene, which encodes a chaperonin that functions in protein folding mechanisms (Mayer and Bukau, 2005). The *hsp70* promoter activates transcription when temperatures are raised 10-15 °C above ambient and has been used in a variety of model organisms to analyze gene function at different times in the life cycle (e.g. Michiue and Asashima, 2005; Ramos et al., 2006; Romano et al., 2001; Wheeler et al., 2000). Global heat shock provides temporal control of gene expression (Shoji and Sato-Maeda, 2008), but does not allow the spatial control necessary for the analysis of region, tissue, and cell specific gene function. Thus methods for local tissue heating would add a powerful tool for the study of gene function in different tissues throughout the life cycle.

Laser-based techniques for controlled heating in small regions of biological samples are desirable, mainly because of the possibility for precise targeting of laser light through a microscope. The most straight-forward approach uses existing microscopy setups to tightly focus laser light onto tissue to induce local heating (Halloran et al., 2000; Ichikawa et al., 2007; Liu et al., 1995; Ramos et al., 2006). The main drawback of this technique is the low absorption coefficient of biological tissues in the near-infrared wavelength range (close to that of water at $\sim 1 \text{ cm}^{-1}$), where typical solid state or diode lasers operate. For example, within 10 μm penetration depth only 0.1% of the laser power is deposited. To achieve temperature differences of 10-15 $^{\circ}\text{C}$, a typical laser with power in the Watt range is required that comes at a significant cost. In addition, precise control and calibration of the temperature is difficult, since exact laser light absorption of biological materials is not well known and random light scattering in biological samples affects the laser power at the target location. A solution for achieving heating with lower laser powers has been proposed by Zondervan et al., who used a metallic substrate that strongly absorbs laser light to heat the environment (Zondervan et al., 2006). However, this approach is not very practical for biological studies as it does not allow three-dimensional positioning of the local heat source. Recently, an infrared laser was used to locally activate *hsp70* transgene expression in *C. elegans* (Kamei et al., 2009). This method is effective, but has similar drawbacks, including difficulties in calibrating heating temperatures and high cost.

The zebrafish has emerged as a powerful genetic system for the study of vertebrate development, with the accessible embryo possessing many features that allow real-time observations and experimental manipulations. The zebrafish *hsp70* promoter

has been used to temporally regulate transgene expression by raising the temperature to 37 °C at any time during development (Shoji and Sato-Maeda, 2008). Both spatial and temporal regulation of a *hsp70:GFP* transgene was demonstrated using a micropoint laser focused through a microscope lens (Halloran et al., 2000). However, this system has had limited utility due to issues of efficacy and cell viability. Spatio-temporal control of *hsp70*-transgene expression was also achieved using a simple soldering iron heating device in zebrafish (Hardy et al., 2007) but spatial control is limited by the size of the soldering iron tip and this system is most useful for gene activation in superficial cells. In addition, the high thermal mass of this device necessitates general tissue cooling using a reservoir.

Our aim was to develop a simple micro-scale optical heater that would allow reproducible and convenient local heating anywhere in the zebrafish embryo without causing cell death. By combining an optical fiber based approach with a low-power laser source, we created an inexpensive local heat source that has a well-defined temperature and can be precisely positioned in biological tissue. Moreover, the area of local heating can be controlled by “pulling” optical fibers with a standard electrode puller, and these fine tips can then be positioned almost anywhere in the embryo or larvae. The temperature at the fiber tip was precisely measured by a thermocouple-based thermometer. This microheater is simple, highly controllable, and induces gene expression without causing tissue damage or cell death. The ability to precisely control heating on the micrometer scale will have broad applications for the study of gene function and may have wide-ranging utility in the fields of medicine and materials science.

Results

A simple optical fiber micro-scale heater

Optical fibers were first demonstrated in the late 19th century, and then mainly used for internal illumination in medical applications, until in the late twentieth century optical fibers became ubiquitous as a medium for telecommunications. Micron-scale optical fibers are now commercially available, allowing delivery of laser light on a biologically relevant size-scale. In our application, a 50 μm core / 125 μm cladding multimode fiber serves as a flexible support and controllable heat source when coupled to a low power laser, namely a 75 mW laser pointer (Fig. 2-1A,B). After removing the fiber jacket, the tip of the fiber is coated with black ink that absorbs the laser light and converts it into heat. We found that $\sim 70\%$ of the laser light is absorbed by the coated fiber optic tip. Such high absorption allows our device to operate with significantly lower laser powers than laser heating devices that use the absorption of the biological sample.

To analyze the functionality of our optical microheater, we calibrated the temperature of the coated fiber using a thermocouple-based thermometer (Fig. 2-1C). The power to the laser was adjusted using a variable current source. We determined that the temperature increased linearly with laser power over a large temperature range with an inverse slope of ~ 0.43 mW/K. Hence, a laser with a power output in the low mW range is sufficient to establish temperature differences that are relevant for biological studies (5-15 $^{\circ}\text{C}$). A current of approximately 55 mA resulted in a temperature of ~ 40 $^{\circ}\text{C}$ which was used for the following experiments.

Local gene activation in zebrafish

To test and optimize the local heater we used the *Tg(hsp70:GFP)* transgenic zebrafish in which expression of the fluorescent protein GFP is controlled by the *hsp70* promoter. The coated optical fiber was brought into contact with an anesthetized zebrafish embryo or larva that was mounted in a drop of 1% low melting temperature (LMT) agarose (Fig. 2-1A inset). The tip was left in contact with the specimen for 20-25 minutes. Embryos were left mounted to monitor GFP gene expression using a dissecting fluorescent microscope. GFP expression was detected 3-4 hours after local heat shock (Fig. 2-1D-F). Embryos were then freed from the agarose and incubated at 28.5 °C in embryo medium until the desired stage.

To vary the area of gene activation, optical fibers of different sizes were made by “pulling” the fiber on an electrode puller. The tapered glass could then be broken to the desired size, from 20 to 120 μm in diameter (Fig. 2-1D-F). An un-pulled fiber tip activated gene expression in a trunk region corresponding approximately to its 125 μm diameter (Fig. 2-1D). Smaller tips activated gene expression in correspondingly smaller regions (Fig. 2-1E,F). Mock treatment, in which transgenic embryos were mounted in LMT agarose and touched with the end of the optical for up to 1 hour with no laser light, had no effect on transgene expression (Fig. 2-1G).

To examine whether different tissues responded similarly to local heat shock, we heated regions of the head and trunk in *Tg(hsp70:GFP)* embryos and larvae of different ages (Fig. 2-2). Transgene expression could be precisely activated in the eye, lateral hindbrain, midbrain, and trunk of embryonic or larval zebrafish (Fig. 2-2A-E). Over the course of these experiments the rate of transgene activation was ~85% (61/72 embryos

heat shocked), through an age range from 10 hours to 7 days post fertilization. This rate approached 100% as we gained proficiency with the apparatus. Local heating in the trunk induced GFP expression in differentiated muscle fibers and differentiated neurons of the spinal cord, with cellular morphology suggesting these cells were healthy and undamaged by the heat shock (Fig. 2-2B,C). The GFP protein is quite stable, allowing labeled muscle fibers to be identified up to 6 days after heat shock (data not shown). “Pulled” fibers in the 10-50 μm range were small enough to allow for penetration into embryonic tissue, making it possible to activate gene expression in deep regions of the brain (Fig. 2-2D). Penetrating with the glass fiber caused remarkably little damage to brain tissue, and no transgene activation was seen along the path of entry.

We also determined the depth of local heating. We calculated that the heating power at the ink coating in water is $\sim 0.26 \text{ mW/K}$, accounting for reflection losses in the lens, fiber coupling losses, and absorption of the ink coating. Considering a simple model of a one dimensional steady state heat flow, we used Fourier’s law of heat conduction to calculate the depth of tissue heating (see methods). Since *hsp70* transgene expression is induced in zebrafish when $T > \sim 37^\circ\text{C}$, the thickness of the expressing tissue was calculated to be 24 μm for a 41 $^\circ\text{C}$ fiber tip. Our experiments under the same conditions show that the thickness of expressing tissue is between 20-30 μm (Fig. 2-2E), in reasonable agreement with our calculations. The thickness of expressing cells could be increased to $\sim 35\text{-}60 \mu\text{m}$, by increasing the tip temperature, consistent with the trend of the model.

Local *hsp70:GFP* transgene activation as a method for lineage tracing

The stability of the GFP protein, combined with the ability to label small numbers of cells, led us to investigate whether the local activation of GFP with this device would provide an easy method of lineage tracing in older embryos and larvae. Other optically based lineage tracing methods such as those using caged fluorescein (Kozlowski and Weinberg, 2000) or the photoconvertible Kaede protein (Hatta et al., 2006) have considerable drawbacks, including the inability to perform subsequent labeling experiments to identify unique cell fates. The GFP protein can be detected with antibody labeling and this labeling is preserved through standard in situ hybridization protocols (see Fig. 2-3D), allowing double or triple labeling strategies to define gene expression in GFP-labeled cells.

Our lab is interested in understanding early pituitary development in zebrafish (Guner et al., 2008; Sbrogna et al., 2003). The anterior lobe of the pituitary, the adenohypophysis, forms as a cranial placode and has been shown to derive from cells at the anterior margin of the CNS in both teleosts (Dutta et al., 2005) and mammals (Kouki et al., 2001). Cells at the anterior neural ridge (ANR) comprise an equivalence domain, and can form either lens or pituitary tissue depending on whether they receive Shh signaling from the CNS at a critical time in their development (Dutta et al., 2005; Guner et al., 2008; Sbrogna et al., 2003). As a proof of principle for local heat shock-based fate mapping, we heated a small region of the ANR at 13 hpf, activating GFP expression in 6-10 cells within 4 hours (Fig. 2-3A). 10 hours later, labeled cells were seen in the developing pituitary placode, as well as in the lens (Fig. 2-3B), consistent with a previous fate mapping study in zebrafish (Toro and Varga, 2007). Another 10 hours later, this

embryo was fixed and labeled using the anti-GFP antibody (Fig. 2-3C). Labeled cells and their progeny were clearly seen in the adenohypophysis and adjacent hypothalamus (Fig. 2-3C'). To demonstrate that the final fate of GFP expressing cells could be determined using this method, we employed the *Tg(pomc:GFP)* line, in which GFP is expressed in POMC cells of the pituitary, but not hypothalamus (Liu et al., 2003). As expected *pomc* ISH labeled all hypothalamic and pituitary *pomc* expressing cells, while only pituitary cells expressing GFP were labeled with the anti-GFP antibody. This clearly shows that ISH/IHC double labeling protocols will allow unambiguous identification of cell fates in the lineage tracing experiments. This method can be adapted for any age and tissue, providing a simple method for fate mapping throughout embryonic and larval development.

Discussion

We have developed a simple laser-pointer driven optical heater that allows reproducible local heating on the 20-150 micron scale. The optical microheater system is comprised of a low voltage power source connected to a laser pointer whose red beam is transmitted along through a lens and optic fiber core and is absorbed by black ink to produce heat (Fig.2-1). Local heating thus occurs at the tip of an optical fiber, whose temperature can be controlled by adjusting laser power and can be precisely measured by a thermocouple-based thermometer. The heated area is mainly determined by the area of the optical fiber as has been shown in Fig. 2-1D-F. This optical local heating system is easy to operate and provides efficient localized heat without general heating of the embryo.

To calculate the potential depth of gene expression we use a simple model of a one dimensional steady state heat flow based on Fourier's law of heat conduction:

$$P = -A\kappa \frac{\Delta T}{L}$$

, in which P is the thermal power, A is the heated area, κ is the thermal conductivity, and ΔT is the temperature gradient over the distance L . With this model we can calculate L (total heated depth) and estimate the depth of gene activation. We approximated zebrafish tissue as water ($k_{water} = 0.6 \text{ Wm}^{-1}\text{K}^{-1}$) and assumed the heated area to be the total fiber tip surface including core and cladding. From the measured temperature of the ink coating as a function of absorbed laser power, we determine $P/\Delta T = 0.26 \text{ mW/K}$. Since 38% of the heat flows into the fish (62% into the optical fiber), we use $P/\Delta T = 0.098 \text{ mW/K}$ to obtain the distance $L = 75 \text{ }\mu\text{m}$, over which the local temperature decreases to the global temperature of the fish (28.5 °C). Knowing that *hsp70* transgene expression is induced in zebrafish when $T > \sim 37 \text{ }^{\circ}\text{C}$, the thickness of the expressing tissue is calculated to be 24 μm for a 41 °C fiber tip. Our experiments under the same conditions show that the thickness of expressing tissue is between 20-30 μm (Fig. 2-2E), in good agreement with our calculations. The thickness of expressing cells could be increased to $\sim 35\text{-}60 \text{ }\mu\text{m}$ by increasing the tip temperature, consistent with the model.

Using zebrafish embryos and larvae we have shown that this new optical heater can reproducibly and safely heat living tissues without causing cell death. This device will have broad application for studies of gene function in living tissues, as it allows precise temporal and spatial regulation of heat-shock responsive transgenes (Fig. 2-2). Local heat shock can be performed multiple times on the same embryo without causing global gene induction in the embryo (data not shown). The area of heating can be

controlled by “pulling” optical fibers with a standard electrode puller, providing a great deal of flexibility in targeting gene activation domains. This precision of gene activation, in combination with the longevity of the GFP protein, provides a simplified method for lineage tracing (Fig. 2-3) that would be particularly useful for the study of late stages of organogenesis, as well as the regulation of cell numbers and fates post-embryonically. Finally, this device can be used to locally ablate cells if higher temperatures are applied. This microheater is simple, highly controllable, inexpensive, and induces gene expression without causing cell death.

Besides the demonstrated uses in embryology, this micrometer scale heater could have broad application for a variety of research and therapeutic purposes. In medicine, local heat-shock inducible promoters could be used to directly activate therapeutic genes (Rome et al., 2005). Local heating could also be used to specifically kill diseased tissue, to locally activate thermally sensitive therapeutic compounds, or to control the innate cellular heat shock response in selected regions. Direct local heating to human tissues can be used in treating cancer (Babbs and DeWitt, 1981), cardiac diseases (Rappaport, 2004), and would be useful in recruiting capillaries in a tissue (Gong et al., 2006), to name a few potential uses. This optical fiber based heater could be easily used to target these treatments more precisely using well-developed arthroscopic procedures. Micron-scale heating can also be used for material science applications to control or catalyze chemical reactions, allowing polymerization of plastics, or creating local melting conditions.

Methods

Microheater components

A 75 mW red beam laser pointer (Pulsar P75 Wicked laser) was connected to a variable power source (HP 6218C Power Supply 0-50V/0-.2A) to control heating. Voltage was kept at 3V while the current was adjusted from 0-150mA. The laser was attached to a mount (Astro-1) on a miniature breadboard (Thorlabs, *MB8 - Aluminum Breadboard, 8" x 8" x 1/2", 1/4-20 Threaded*). A focusing lens (Thorlabs, focal length 4 cm) was placed in a translating mount (Thorlabs, LM1XY) in front of the laser pointer. The end of a ~13" long, 50 μ m core optical fiber (AFS50/125Y 0.22-NA 50 μ m Core Multimode Vis-IR Fiber) was placed in a fiber clamp (Thorlabs, HFF003) approximately 4 cm from the lens. The fiber clamp was mounted on a translation stage (Thorlabs, MS1) to adjust the distance between the fiber end and the lens. As desired, optical fibers were pulled on Sutter Micro-Pipette Puller (Model P-97). Before pulling, the fiber optic coating was removed from a small region by flaming briefly. The bare end of the fiber was coated in permanent black ink, taped to a wooden dowel, and mounted on a Narishigi Micromanipulator. Temperature at the fiber tip was adjusted by touching the tip to a k-type thermocouple (0.0005" diameter, Omega CHAL-0005) attached to a digital thermometer (Omegaette HH303 Type K J Thermometer). The thermocouple was mounted on plastic to prevent breakage and facilitate probe calibration. Current was adjusted to achieve a tip temperature of about 38-40 $^{\circ}$ C (~55 mA depending on tip size). The temperature of the optic fiber tip was recorded before and after local heat shock.

Zebrafish mounting and local heat shock

Tg(hsp70:GFP) transgenic zebrafish lines (Halloran et al., 2000) were maintained as previously described (Westerfield, 2000). Transgenic embryos were grown in Embryo Raising Medium (ERM; 2M MgSO₄, 2M KCl, 2M CaCl₂, 5M NaH₂PO₄, 5M NaCl; (Westerfield, 2000) at 28.5 °C. For heat shock, dechorionated embryos or larvae were anesthetized using MS-222, embedded in a drop of 1% Low Melting Temperature (LMT) Agarose (Sigma) on a petri dish, and positioned using forceps before the agarose set. The coated tip of the fiber optic was brought into contact with the tissue and heated for 25 minutes. For deep tissue heating, 30-40 µm diameter fibers were pushed into embryonic tissue. As needed, skin over the heating site was disrupted using a sharp probe or by short exposure to a small drop of mineral oil. Embryos were kept mounted for 2-4 hours after heat shock and gene activation was documented on a fluorescent dissecting microscope. Embryos were freed from the agarose and incubated in ERM at 28.5 °C. The fiber tip was recoated with ink after 7-10 uses, as needed.

Antibody Labeling and Imaging

Embryos and larvae were fixed for 1 hour at room temperature in 4% paraformaldehyde and stored in methanol at -20 °C. Immunohistochemistry was performed essentially as in (Barresi et al., 2005) using a rabbit anti-GFP primary antibody (1:400, Molecular Probes) and a goat-anti-rabbit Alexa Fluor-488 (1:1000, Molecular Probes) secondary antibody. For double in situ/antibody labeling, anti-GFP antibody labeling was performed after fluorescent in situ labeling using the Fast Red fluorescent substrate (Roche). Labeled embryos were cleared and mounted in 75%

glycerol for DIC and fluorescent imaging using an Axioplan 2 compound microscope equipped with the apotome confocal system (Zeiss).

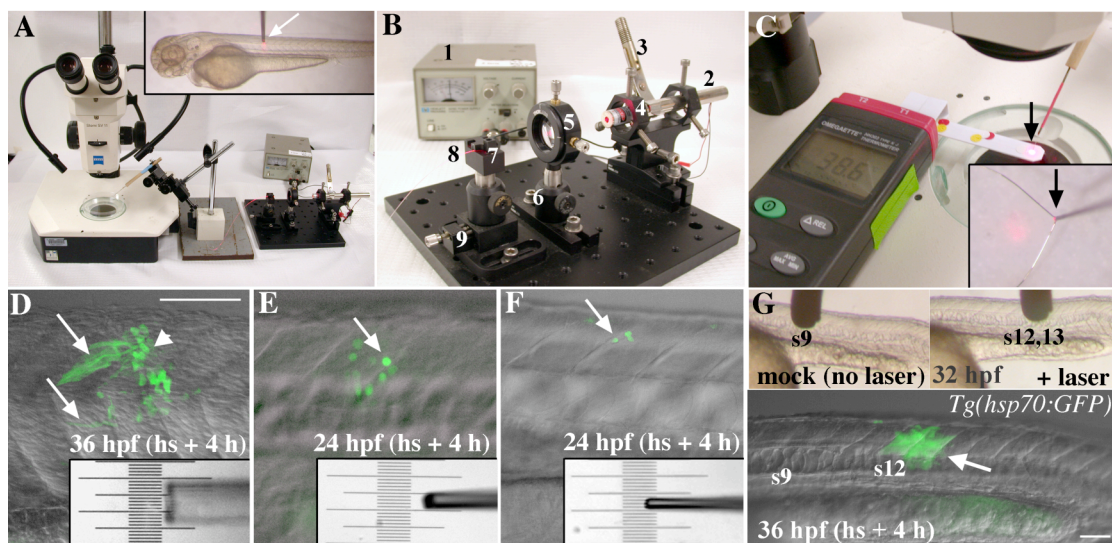


Figure 2-1. The laser-pointer optical micro heater and local *hsp70:GFP* transgene induction.

(A) Laser pointer beam was focused through a lens onto the end of a 13'' long optic fiber. The other end of the fiber was mounted on a micromanipulator and positioned next to the target tissue under a stereomicroscope. Inset shows the heater-end of the optical fiber (arrow) contacting the trunk of a 72 hpf zebrafish larva mounted in agarose. (B) Close-up view of the laser pointer microheater assembly. (1) Variable electrical power supply (2) 75 mW red beam laser pointer, (3) Clamp to depress the laser pointer switch, (4) Hexagonal holder for laser pointer, (5) Lens in translating lens mount, (6) Post holder, (7) Fiber clamp, (8) 50 μ m core optical fiber, (9) Translation stage. The focal length (the distance between the lens and the tip of the optical fiber) is 4 cm. (C) Digital thermometer used to set the temperature of the laser tip (arrow) before performing local heat shock. Inset shows close-up of the heater tip contacting a k-type thermocouple (black arrow). (D-F) Examples of local transgene activation in *Tg(hsp70:GFP)* embryos, lateral views of the trunk (see A inset). Somitic tissue was heated for 25 minutes using different sized fiber tips calibrated to 41 $^{\circ}$ C. Insets show different sized tips over a micrometer. (D) GFP expression in muscle fibers (arrows) of a 36 hpf embryo 4 hours after heat shock using the intact 120 μ m diameter (50 μ m core) optical fiber tip. (E) GFP expressing lateral cells (arrow) in a 24 hpf embryo 4 hours after heat shock using a 50 μ m diameter tip. (F) GFP expression in a few lateral cells of a 24 hpf embryo 4 hours after local heat shock using a 30 μ m diameter tip. (G) Mock treatment (optical fiber contact for 25 minutes, but no laser light) in the region of somite 9 (left inset) did not activate gene expression, while local heat shock in the region of somite 12 (right inset) in the same embryo led to transgene activation in somites 12 and 13 (arrow). Scale bars: D-F, 100 μ m; G, 50 μ m.

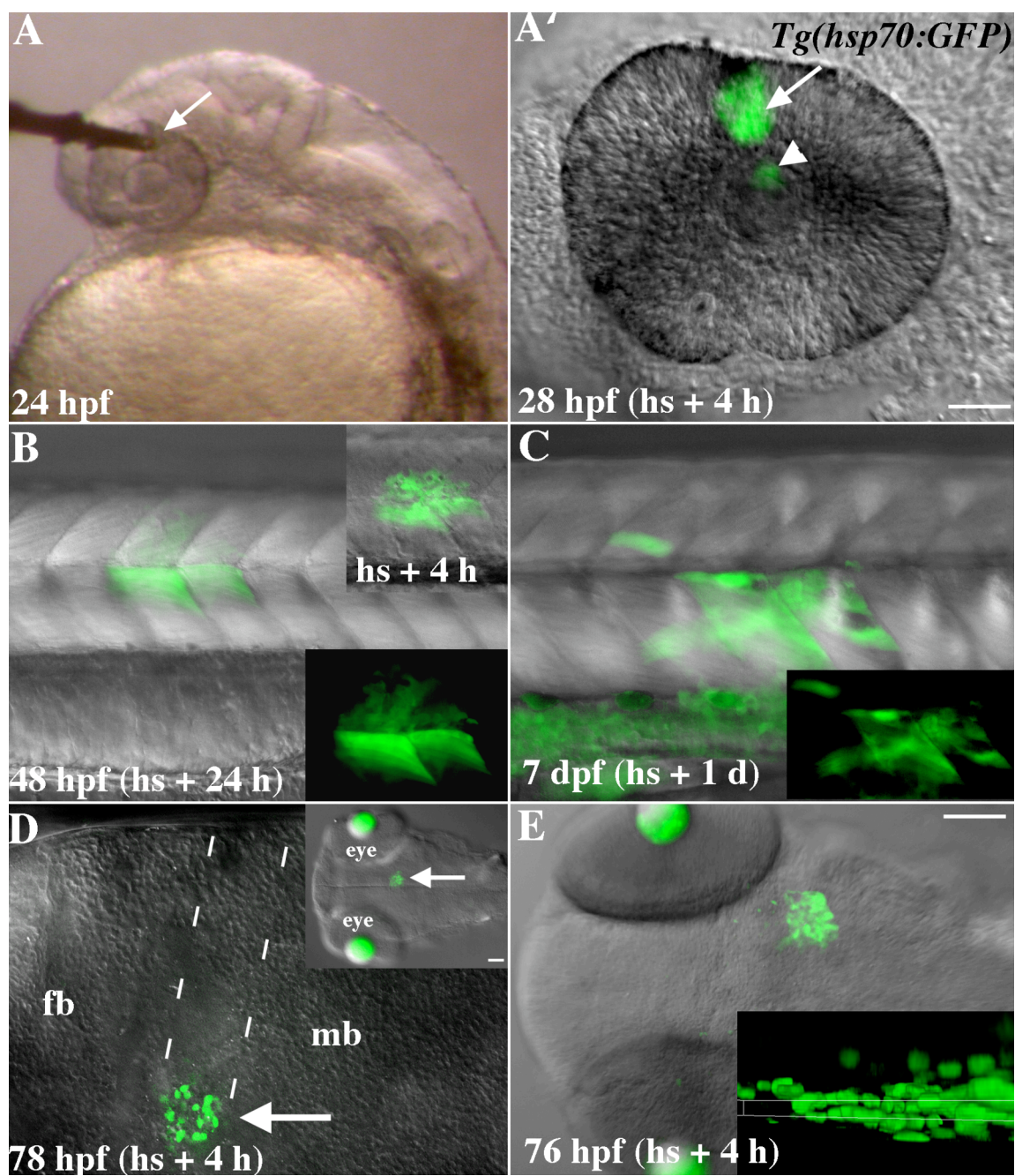


Figure 2-2. GFP expression in *Tg(hsp70:GFP)* embryos following local heat shock.

A) A 50 μm diameter micro heater tip (arrow) touching the surface of the eye of a 24 hpf embryo mounted in low melting temperature agarose. (A') GFP expressing cells (arrow) are seen in the heated region 4 hours later. GFP fluorescence is seen reflected by the lens (arrowhead). (B) GFP expression (arrows) in a 48 hpf transgenic embryo heat shocked 24 hours earlier in the trunk with a 100 μm optical fiber tip. Muscle fiber morphology in GFP expressing cells is normal, indicating these cells were not damaged by heat shock. Upper inset shows GFP expression 4 hours after heat shock. (C) Transgene activation in a 7 day larva that was heat shocked at 6 dpf. Inset with fluorescent channel shows healthy muscle fiber morphology. (D) A 50 μm tip was pushed into the brain of this embryo at 72 hpf to locally heat deep tissue (arrows, dotted lines show entry pathway in this lateral view of the brain, dorsal up). Transgene expression was activated with relatively little damage from the optical fiber. Inset shows the region of gene activation in a dorsal view. Lens tissue fluoresces in this transgenic line at these later developmental stages. (E) Dorsal view of GFP expression in the midbrain of a 76 hpf larva 4 hours after heat shock. Inset shows rotated lateral confocal image, dorsal up. GFP expression was activated in cells ~ 30 μm away from the dorsal surface that contacted the fiber optic tip. Again, lens tissue fluoresces in this transgenic line at larval stages, serving as a useful means of identifying transgenic individuals. Scale bars: A-D, 50 μm ; E, 100 μm .

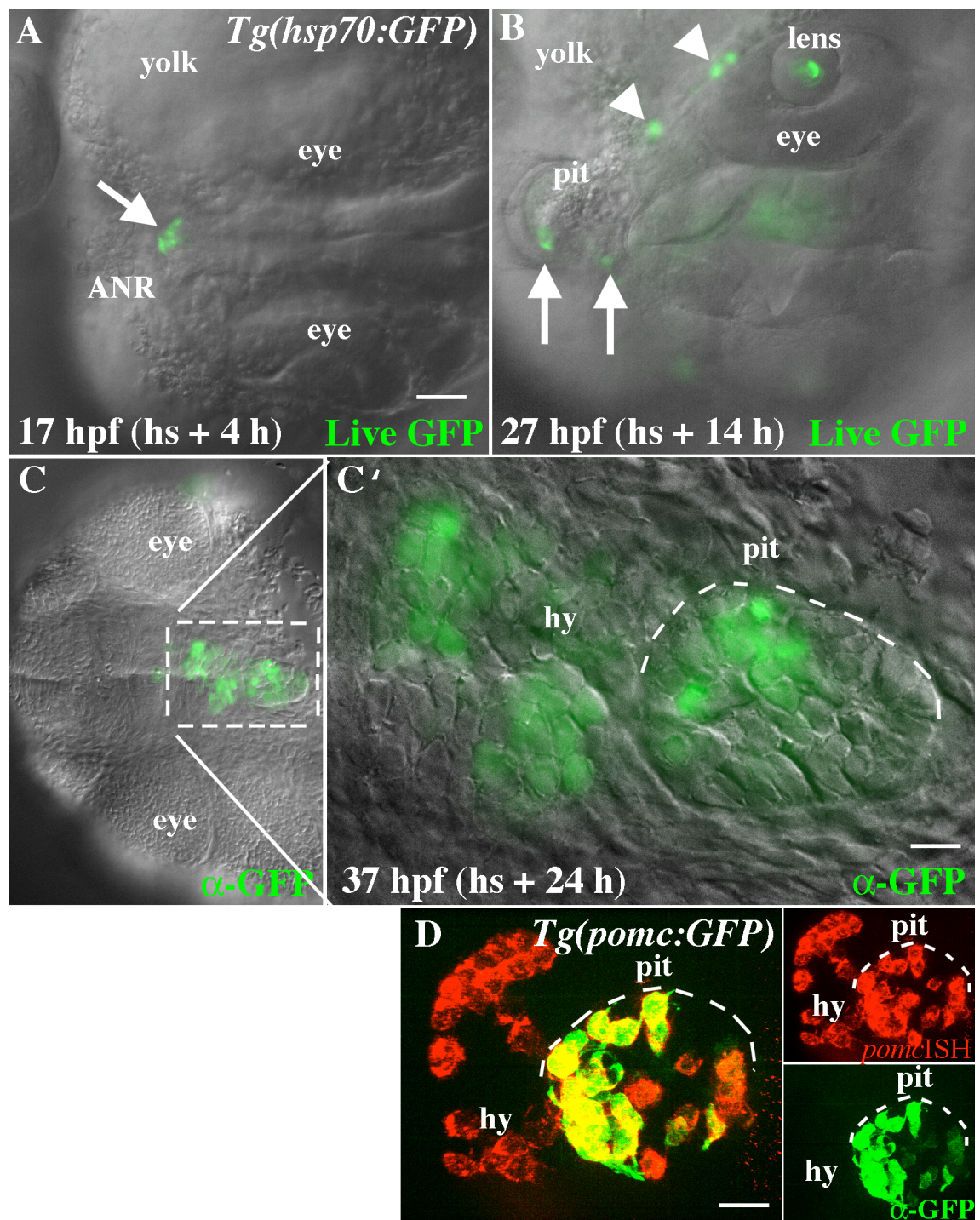


Figure 2-3. Lineage tracing by local activation of the *hsp70:GFP* transgene.

(A) GFP-expression in 4-6 cells at the anterior neural ridge (ANR, arrow) 4 hours after local heat shock. Dorsal view, anterior left. (B) 14 hours after heat shock GFP positive cells were present in known derivatives of this region including the pituitary placode (pit, arrows), marginal epidermis (arrowheads), and lens. (C, C') 24 hours after local heat shock GFP positive cells were seen in the hypothalamus (hy) and pituitary (pit). D) Double in-situ/antibody labeling illustrating the ability to label GFP positive cells following in situ labeling of gene expression. In the *Tg(pomc:GFP)* line, *pomc* expressing cells of the pituitary express GFP (green label), while *pomc*-expressing hypothalamic cells do not express GFP (Guner et al., 2008; Liu et al., 2003). Hence only pituitary cells are labeled by the GFP antibody in this line. Fluorescent in situ hybridization using a *pomc* antisense RNA probe labels both *pomc*-expressing populations (red). Scale bars: A-C, 50 μ m; C', 10 μ m; D, 20 μ m.

CHAPTER III

DETAILED HEAT SHOCK APPARATUS PLANS, MATERIAL, AND METHODS

Laser-Pointer Microheater assembly

Component List

- (a) Power Supply (HP 6218C Power Supply 0-50V/0-.2A, Hewlett-Packard)
- (b) 75mW Red Beam Laser Pointer (Pulsar P75 Wicked laser)
- (c) Hexagonal holder for laser pointer (Astro – 1, Beam of Lights Technologies)
- (d) (1x) Post Mounted Translator (LM1XY Thorlabs),
- (e) (1x) Post Mountable Fiber Clamp 250 μ m (T711-250 Thorlabs),
- (f) 50 μ m core Optical Fiber (AFS50/125Y 0.22 -NA 50 μ m Core Multimode Vis-IR Fiber, Thorlabs) (10 m spool, use 13 cm lengths with scissors)
- (g) Half inch posts and post holders (TR and PH-ST series, Thorlabs)
- (h) Translation stage (Thorlabs MS1),
- (i) 1/4"-20 kit (e.g. HW-KIT2, Thorlabs),
- (j) (1x) Miniature Breadboard (MB8 - Aluminum Breadboard, 8" x 8" x 1/2", 1/4-20 Threaded, Thorlabs)
- (k) (3x) BA 1 regular bases (Thorlabs). The focal length (the distance between the lens and the tip of the optic fiber) should be 4cm (1.5"). (see Fig.3-A):

Note: Order laser from www.wickedlasers.com, Mount parts from: www.thorlabs.com

Connect laser pointer to adjustable power supply and mount on a mounting plate

1. Replace the batteries of the 75mW laser with a piece of wood with wires to make contact between the power supply and the electrodes of the laser.
2. Connect the wires to the power supply and adjust the voltage to 3V (The power should be off at this time). Anything less or more than 3V could burn the 75mW laser diode.
3. Mount the 75mW laser on its holder base (C,K) and use a clamp to depress the laser pointer power switch.
4. Mount lens (D) and fiber optic holder (E) on adjustable mounts as shown.
5. Make sure the voltage is set at 3V, don't go over this or you will blow out the laser point. Current will be adjusted to obtain the desired temperature. Start with approximately 50 mA.

Note: You will need to keep the laser-pointer power button depressed and turn the laser ON and OFF from the power supply (ON/OFF button)

Digital thermometer Assembly

Use a K-type thermocouple (t/c) (diameter .0005") (Omega; CHAL-0005) and a k-type male plug (Omega; HFMPW-K-M). The k-type thermocouple comes un-insulated on a white plastic holder and the ends are held together by red (positive end) and yellow (negative end) tape. (see figure Fig. 3-2)

1. Shorten the plastic holder so it can be mounted on the digital thermometer. To do this, lift the red and yellow tape with forceps to lift the T/C wires, leaving the other end as is (the heat detector). Cut the plastic 10 cm from the heat detector. Keep the cut end for the next step.
2. Glue the plug to the cut end of the plastic with superglue. To do this, place the back side of the male plug on the glued portion of the plastic holder. Screw the negative end (yellow tape) of the thermocouple to the positive terminal on the plug, and the positive end of the thermocouple (red tape) to the negative end of the male plug (yes, this is backwards). Use tape to prevent the wires from contacting each other. Screw the plug together, and glue the cut end of the plastic on top to help protect the wires.
3. Plug this unit into the digital thermometer (OMEGAETTE HH303 Type K J Thermometer). Turn the digital thermometer on for several minutes and verify that it correctly measures room temperature.

Optical Fiber Preparation

1. Remove the coating from both ends of the 13cm optical fiber by flaming using a cigarette lighter. Remove ~ 5 cm of coating from one end and ~ 2cm from the other.
2. Use forceps to make a clean, perpendicular break at both ends. Check under a dissecting scope.

3. Coat the longer end of the fiber with permanent black ink. An easy way to do this is to extract the ink from a black Sharpie® marker using a 27 gauge needle and a 3mL syringe. Allow the ink to dry on the fiber for approximately 5 minutes.
4. Tape the coated end of the fiber optic to a ~0.5 cm wooden dowel (a dissecting probe handle works well) to mount on a micromanipulator.
5. Place the non-coated end of the 13cm long optical fiber in an adjustable clamp approximately 4cm from the lens, leaving ~ 2mm overhanging.

Optional: an electrode puller can be used to produce smaller diameter tips

1. Remove the coating from a ~4 cm region near one end of the 13cm fiber optic by quick flaming.
2. Pull the optical fiber with a Sutter Micro-Pipette Puller (Model P-97) using the following parameters: Heat 640 mA/sec, Pull 120, Velocity 200, Time 200 seconds.
3. Pulled optical fibers are usually 10 micrometers in diameter. We used forceps and break the tip to the desired size.

Calibrate/Adjust the temperature at the microheater tip

1. Place one or two drops of water top of the k-type thermocouple (the little ball). Using the micromanipulator, gently touch the coated tip of the fiber to the t/c.
2. Optimize the temperature obtained at the tip by focusing the laser light on the uncoated end of the fiber (move the lens in the x/y direction), and by adjusting the distance between the lens and the uncoated fiber. Once the maximum

temperature is achieved, adjust the current to produce a temperature of $\sim 40^{\circ}\text{C}$.

For our setup, $\sim 55\text{mA}$ produces the desired temperature.

Notes: The coated optical fiber tip must touch the heat detector in the middle. Avoid vibration, you don't want to scratch the ink off of the tip.

Embryo Mounting and Local Heat Shock

1. Mount anesthetized embryos or larvae in a drop of $\sim 1\%$ Low Melting Temperature (LMT) Agarose (Sigma) on a slide or Petri dish. Position as desired before the agarose hardens.
2. Touch the calibrated fiber tip to the embryo. Contact needs to be made, but keep it gentle.
3. Turn the power supply ON, and laser heat shock for 25 minutes at 400°C .
4. Embryos can be left in agarose to monitor transgene expression (e.g. GFP fluorescence). Cover with ERM so they don't dry out. Alternatively, embryos/larvae can be freed immediately.

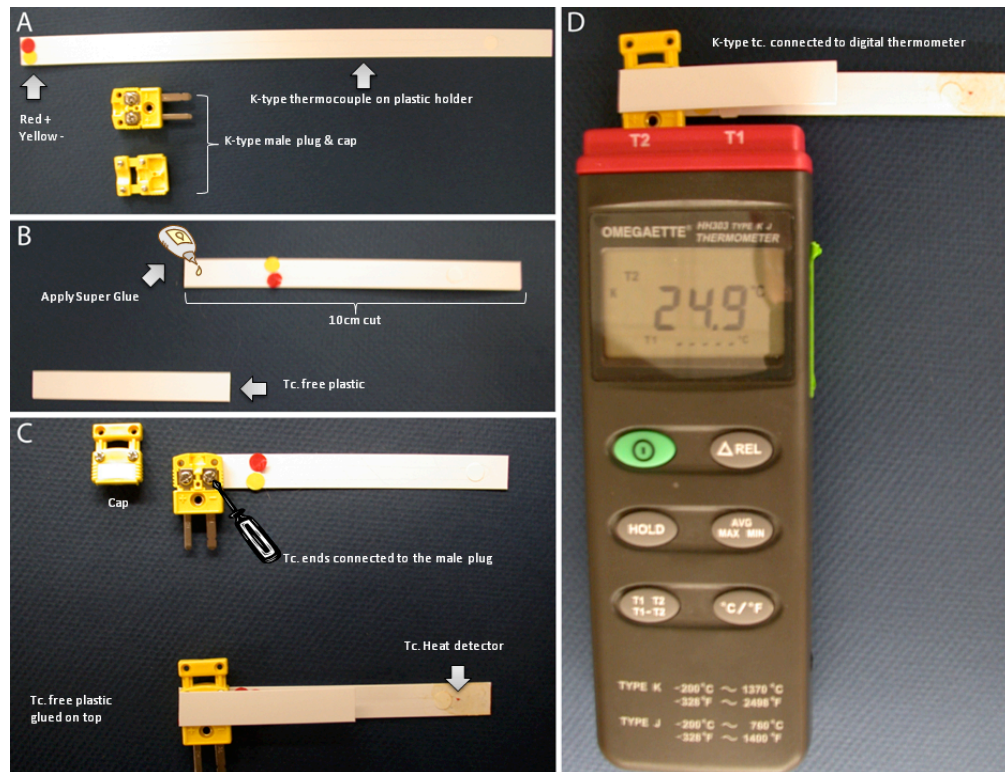


Figure 3-1. Digital Thermometer Assembly.

A-C shows the K-type thermocouple connected to a male plug, which was initially inserted into the digital thermometer (D). The bead of the thermocouple found at the junction of the thermocouple wires gives accurate temperature reading.

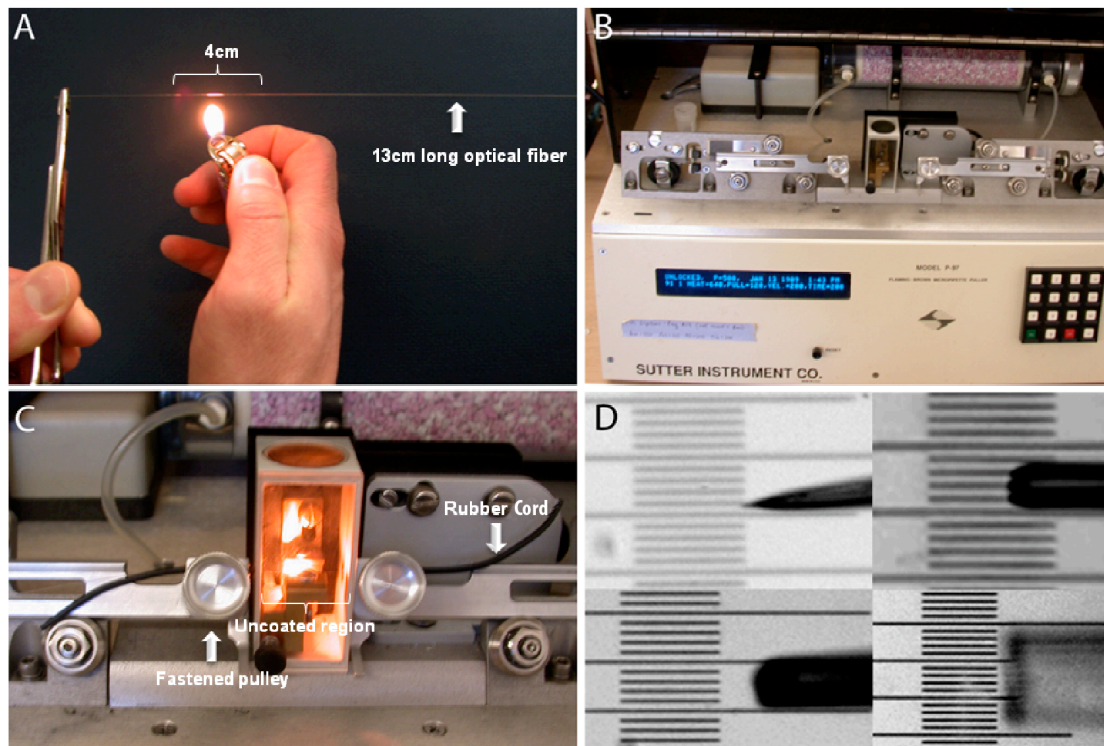


Figure 3-2. Making different size optical fiber tips.

(A) applying flame to a small portion of the optical fiber end, (B) the electrode puller's parameters for optical fiber pulling were found and (C) the un-coated region of the optical fiber was placed adjacent to the heating element, allowing for the generation of small optical fiber tips. Forceps were used to change the diameter of the tip by breaking the tip under a micrometer (D).

CHAPTER IV

GENERAL DISCUSSION

Overview of new local heat-shock apparatus

We have now developed an effective device for local tissue heating in zebrafish embryos and larva. Local gene activation has been accomplished in the past in zebrafish using a modified soldering iron tool (Hardy et al., 2007) or an expensive laser that induces gene expression in single cells (Halloran et al., 2000). In *C. elegans*, local heating was achieved using a 560 nm laser device with positioned mirrors (Ramos et al., 2006), or an IR laser device that was used to irradiate cells where GFP expressing *E.coli* were used as the thermometer (Kamei et al., 2009). None of these tools provide precise regulation of heat at the tip, nor are they as simple or fast to use as our 75 mW optical laser device. Our results show that optical heating locally activated gene expression in 61 of 72 transgenic embryos tested (Table 1-1) over the year of experimentation. As we gained proficiency with the tool our success rate has reached 100%.

Our innovative laser tool offers good control of gene activation, without using any thermal cooling system (Hardy et al., 2007), and may become useful to provide fine spatial control of gene activation for example under the control of the GAL4-UAS system, using a heat inducible promoter such as *hsp70*.

Comparison of Wire and Laser/fiber-optic methods of local heating

We initially built a local heater using micron-scale wires with an Omega current controller in collaboration with Mike Conboy, the UMass Chemistry Department Electrician. This device, while functional, proved to be difficult to control and required high maintenance since the heating wire broke with every few uses. In collaboration with Marc Achermann of the Physics Department we turned to the optical microheater that is the focus of this thesis. When it comes to precise control of temperature at the tip, the optical laser device was superior for several reasons: it was fast and easy to setup. The optical microheater tool gives a more restricted heat shock area, offers very precise heat control at the tip, and reduced tissue damage and cell death. Moreover, the benefit of using our laser technique is that there is no need to fabricate new heating tips for the optical laser, since the tip itself is an optic fiber that can be recoated indefinitely,

The control of temperature at the tip of the wire microheater was problematic. Each time we tested for the temperature of the wire tip using the Omega digital thermometer the temperature reading fluctuated over a wide range (sometimes by 10 and 20 degrees Celsius). This fluctuation in temperature was observed in every wire-heating probe that was developed and was most likely due to the high resistance of the wire.

In contrast, controlling the temperature of the 650 nm beam transmitted through the optical fiber was not trivial. The heat adjustment was done by turning the current flow to about 155 mA, and the current needed to maintain a given temperature was extremely consistent over the course of 5 to 7 local heat shock experiments. Before and after every local heat shock experiment, we tested for temperature at the tip of the optical

fiber using the Omega digital thermometer. The temperature reading at the tip differed only by 1 °C

Efficiency and precision of gene activation

Our successful experiments show that the optical laser tool is more efficient in locally controlling gene expression than other local heat shock tools developed up to this point, providing precise heat control at the fiber optic tip with minimal maintenance. It is easy to assemble and the optic fiber does not need to be replaced. It is also safe since it does not require any light adjusting mirrors for laser beam transfer; instead the beam travels directly through an optical fiber. This new system is also simple since the embryo is heat shocked in one or two drops of agarose, and there is no need for any additional medium for cooling.

This optical microheater tool is efficient in activating gene expression in multiple tissues, including the brain, eyes, spinal cord, and trunk muscles. Different diameter optical fiber tips (20-100 µm) can easily control the area of gene activation at any depth and in any tissue type. The diameter of the optic fiber tip is consistent with the heat shocked area; for example a 50 µm optical fiber tip will activate gene expression in cells that lie within the 50 µm field, and the local heat does not induce heat shock genes anywhere else in the zebrafish embryo.

Fate mapping

Fate mapping is a method used to track cell lineages during development. This method allows researchers to see where cells originate and helps define the embryonic environment that guides cell migration and differentiation. Traditionally, fate mapping as a technique has been limited to the early embryo, when it is easier to mark small cell populations, usually by injecting lineage tracing dyes.

Because the laser micro heater is capable of activating GFP expression in *Tg(hsp70:GFP)* zebrafish at late stages of embryonic and larval development, and because GFP fluorescence lasts for many days, we tested whether local GFP activation could be used for lineage tracing/fate mapping experiments. The fact that GFP expressing cells can be photographed without causing damage also allows for time-lapse analysis of cell division and cellular movements. This technique worked extremely well, allowing us to confirm previous fate map data for the anterior margin of the nervous system (see Fig. 2-3). The stability of the GFP protein through the in situ hybridization protocol allows us to determine gene expression in GFP expressing cells. Thus final fates of GFP labeled cells can be determined unambiguously. Because GFP expression can be induced at late larval stages, this technique opens the door for fate mapping studies in different organs/tissues throughout the life cycle. This may be important for understanding the regulation of stem cell proliferation and differentiation in the adult, for example in the nervous system, heart, pancreas, or any other organ.

Other optical techniques for lineage tracing have been developed in zebrafish, but each has its limitations. These include the Kaede photo-convertible protein, fluorescent dextran molecules, and caged fluorescein (Tomura et al., 2008). Fluorescent dextran

lineage tracers must be directly injected into cells, a technique that becomes more and more difficult as embryos develop and may be impossible in adults. Cells that express the photoconverted Kaede protein can only be traced in live embryos, as the fluorescence is lost following fixation. Thus the final cell fate of labeled cells can not be determined using in situ and immunohistochemical analyses of gene expression. Caged fluorescence requires light to activate fluorescence in cells that carry the compound. “Loading” cells with caged fluorescent markers is done by injecting embryos at the 1-2 cells stage, thus dilution becomes a serious issue at later embryonic stages. Further, caged-fluorescence has severe limitations due to spontaneous uncaging and has had limited use, even in the transparent zebrafish embryo.

Surprisingly, there have been no reports describing the use of local heat shock to monitor the movement and fates of cells during development. The conditions described here to induce local gene expression in a small number of cells during zebrafish embryogenesis could be applied to allow fate mapping in other model systems where the genes are under the control of a heat shock promoter. For example the optical microheater could be utilized in fate mapping in any model system where the transgenic line is under the control of a heat shock promoter. Temporal regulation of genes in mouse, zebrafish and other systems for example would allow us understand better specific cell fate commitment such as identifying the neuronal migration during early embryogenesis and therefore aiding in understanding better the diversity of the nervous system. We have activated gene expression in as few as 4 cells, enabling us to follow isolated cell movements and visualize final cell fates at a later time.

Possible improvements

Following local heat shock, it was noted that not all cells in the heat-shocked region expressed GFP, suggesting that the activation of gene expression is mosaic. This is likely due to the minor damage to the ink coating that allows light to pass through the tip instead of heating the tip of the fiber optic. A permanent, non-toxic black tip coating material would solve this problem. One possible solution would be to apply a polymer that has the capacity of fusing or binding permanently to the fiber optic core tip. Ideally this polymer would need to be black and/or be composed of a material that would absorb the radiation from the laser to provide heat.

Study of Late gene function: Shh

The Hedgehog signaling pathway is involved in patterning of different tissue types in early development and continues to play a role throughout the life of the organism, including by regulating proliferation and differentiation of neural stem cell. The lab has developed transgenic lines in which the *hsp70* promoter drives expression of Shh itself, the Gli1 transcriptional activator of Hh signals, or a dominant repressor Gli2 transcription factor that blocks Hh signals. Given these lines have already been generated, the temporal and spatial control provided by our laser pointer tool will allow the lab to investigate cell-autonomous roles for Hedgehog signaling in cell differentiation, proliferation and cancer in larval and adult zebrafish.

Broad Applications for life science research

Local heating can be used for various biological applications, such heating living tissues, aid in deactivating pain receptors due to injury or disease, and it can be used to affect neuronal activity in specific locations since a small change in temperature alters nerve conduction. Direct local heating can also aid in increasing blood flow in small capillaries or to ablate cells in small cancer tumors, which can improve in faster destruction of cancer cells by using lower chemotherapy radiation. Additionally, it can be used on small blood vessels, making them leakier and therefore allowing faster penetration of chemotherapy and can be used in controlling gene expression in combination with a heat shock promoter.

Study vertebrate proton-pump cells in epithelia

Skin plays a vital role in an organism's life, providing protection, temperature regulation and insulation. Since zebrafish are transparent during early development, it provides a great model system to study the secretion of protons and ionocytes in vertebrate's epithelia (Lin et al., 2006). A change in temperature affects the development of fish embryos a lot, and we therefore believe that local heating at the micro-level would aid in studying the physiology of the epithelial cell types that excrete protons in zebrafish during its development. Understanding better the cell types that excretes protons in epithelia would allow us to develop new and better drugs that would control the skin pH such as in aging, since age makes us humans more susceptible to bacterial infections, and other skin diseases.

Lobster Shell Disease

Local heat shock can aid in studying American lobster shell disease. Research has shown that the exoskeleton of the American lobster, found in the waters along the Eastern Coast of America, is dissolving at a dramatic rate during the warmer months (Tlusty et al., 2007). Scientists believe that the increase in the prevalence of shell disease in American lobsters may be linked to global warming. We therefore believe that local heating of the lobster exoskeleton will facilitate the study how proton flux and calcium dependency varies with temperature and pH. This study would be of tremendous help since 50% of the economy in Maine depends on American Lobsters.

Broad Applications for materials science research

Controlling heat at the micrometer level would eliminate the potential of damaging important micro structures that could be critical in microelectronic devices, such as in cooling of microchips. Local heat can be used in making polymer-based micro-sensors that are directly integrated to silicon microchips. These microsensors for example can be useful to monitor small molecules within the brain, or can aid in testing new drugs.

Microchip bonding

Microchip bonding is used in annealing of micro channels within computer microchips. Low heating has been used in the past for polymerization of microchips. When it comes to microchip components there are a whole bunch of tools that have been used and known to affect the integrated circuits within the microchip itself during

bonding. As an alternative, epoxy-free bonding has been used in bonding metallic and non-metallic material components, but this type of bonding is limited to other components that require non-metal bonding. Another major problem with other types of bonding is the spread of heat to the surrounding and so affecting/loosening other micro components within the microchip. An alternative to this problem would be to deliver local heat that is very controllable and could be utilized in low temperature epoxy binding in microchips, which will provide great strength in the bonding process.

Polymerization in microfluidics

Microfluidics is a new emerging field that deals with the study of the control of small scale fluids. These small-scale polymer-based microfluids come in different shapes, and are composed of a wide range of biomechanical properties, such as crystallization of proteins. They are known to be used in biological analysis, nanoparticle synthesis, and many other varieties of medical and non-medical related applications. Fabricating microfluids is a big challenge, especially when it comes to bonding polymers that would hold the microfluid channel structures together. They are fabricated in polydimethylsiloxane and have channels that are disconnected at the micro-level scale. Bonding of channels has been achieved by polymerization with low spread of heat, but the biggest problems that appeared were the deformation of surrounding microstructure (Seger-Sauli et al., 2005). However, applying localized heat with the optical microheater will could allow local optimal bonding strength without distorting the surrounding microscale channels.

Possible Medical applications

Local heating on the micrometer scale has the potential to be very useful in the treatment of a variety of human medical conditions. Heat has been used in battling cancer tumors, treating damaged tissues, and coagulated vascular veins. Microwave and direct laser light radiation heating are some of the tools used in medicine today to deliver local heating in human tissues. However, these tools don't allow direct contact with the tissue, therefore heat is distributed to unwanted regions. The heat generated by microwaves does not penetrate the tissue deep enough, while our optical microheater allows deliverance of thermal energy to any depth within the tissue. We believe that our optical microheater can provide improved heat control and distribution in any tissue type, even a small cardiac artery. For example, many cardiac surgeries today rely heavily on 'balloon angioplasty', which allows access to the arterial plaque. One of the major problems that occur with this procedure is a widening of the arterial walls that eventually affects the blood flow after the surgery. However, if using a small diameter microheater, such as our optical microheater, there will be no need to widen the blood vessels; instead controlled heating to the clogged artery could be used to melt the plaque, without damaging the blood vessel. Similarly, new studies have shown that heating could be used to impede tumor growth in different tissue types, and also to relieve certain kinds of pain. We believe that our optical microheater could be also used for such medical applications, and would greatly allow avoidance of unnecessary cell heating surrounding the target cells that may exert negative effects on other cells.

Our tool could be of great use in studying the effect on temperature on ion flux out of the bone in vertebrates under different temperature conditions. Studies have

demonstrated that bone constantly exchanges ions and that the calcium influx in the bone relies a lot on the external conditions (Marenzana et al., 2005). However, upon injury or bone disease, the ion influx is affected, leading to problems in maintaining a proper ionic complement in the bone. Using a local heating device would be very helpful to look at the calcium flux in people that have bone diseases in specific and small areas. In order to get heating at the depths required to heat bones, this study could be done using more superficially located bone structures such as fin rays.

Final Conclusions

We demonstrated that we can induce local transgene expression with our innovative optical microheater in the zebrafish embryos. The optical microheater can be used to study gene function in many different organisms at any time in the life cycle, or for fate mapping studies at any stage in organogenesis. This optical technique of local heating is fast and it could be used for some of the most complicated known surgical procedures in humans, such as open heart surgery, and treating various human diseases.

The direct local heating can aid micro-technology, such as in the construction of more complex microchips for better electronics, that are smaller in size and have a longer lifetime. The optical microheater can be used to form small structural channels through catalysis of bonding polymers. These small channels would allow tiny volumes of fluids to be used as cooling devices on microchips, making them more energy efficient. The optical heater can be used in bio-electronic technology that is mainly based on microchips and sensors. This technology is now being developed for identifying the risk

of thrombosis in humans and also to create new artificial biomembranes. Our tool could therefore be used to manipulate and/or construct these artificial membranes.

Another possible future direction would be to test the effect of thermal energy of different color coatings that could probably be used for specific applications in different organisms.

Table 1-1. Laser Local Heat Shock.

Embryo age (hpf)	Region	# Heat Shocked	# + GFP
8-16 somites	Trunk	14	11
14 -16somites	ANR	10	8
16 somites	Trunk	2	1
24hpf	eye	2	2
24hpf	Trunk	5	4
28hpf	Trunk	1	1
30hpf	Trunk	2	1
36hpf	Trunk	10	9
48hpf	Trunk	8	8
48hpf	Eye	1	1
48hpf	Brain	2	2
52hpf	Trunk	8	6
72hpf	eye	1	1
72hpf	trunk	1	1
76hpf	trunk	1	1
4daysphf	Trunk	2	2
7days hpf	Trunk	2	2
Total	-	72	61

ANR is the anterior neural ridge of the zebrafish embryo. The # Heat Shocked means the number of transgenic zebrafish embryos (*hsp70:GFP*). The #+GFP means the number of embryos expressing GFP after local heat shock.

APPENDIX

A WIRE MICRO-HEATER FOR CONDITIONAL REGULATION OF GENE EXPRESSION IN ZEBRAFISH EMBRYOS

Introduction

Global heat shock is known to activate transgene expression throughout the embryo under the control of the *hsp70* promoter, therefore allowing temporal regulation of gene expression in the zebrafish embryo. My primary goal was therefore to develop micro-wire heating tips that would allow both temporal and spatial regulation of genes in the zebrafish embryo at any time during development, and find the optimal heat shock temperature of the tip by testing the newly made probes on transgenic embryos (hsp-GFP). The micro-wire probe would be connected to a digital control device that would regulate the heat at the tip of the wire probe. The tip of the wire probe employs a very small nichrom wire that has a diameter that is not large and doesn't increase in thickness as the tip of a soldering iron tool (such as the one used by Chi-Bin Chien) therefore allowing us to exclude the use of cooling medium.

Methods

Wire-heat shock apparatus setup

Figure A-1 details the setup of the wire heat shock device. The custom made microheater was connected through wires (igniter wire and thermocouples) to the input and output of the control box (Fig.A-1A). We fixed the body of the microheater into the micromanipulator (Fig.A-1B) to enable us to position the wire at close proximity to the embryo, without damaging the embryo (Fig.A-1C). The Control Box (a white plastic box

10''x 6'') consists of a digital controller (Omega CNi16D43) (Fig.A-1D), a 5V DC power supply (Fig.A-1E), a solid state relay 1.8A (max) (NEC PS710A-1A) (Fig.A-1F), a 1.25A current limiter (LM317 Regulator) which will protect the power supply and the solid state, an AC power switcher, and a heat indicator. The digital controller was manually configured for the J-type thermocouple and auto-tuned. The tip of the heating probe needed to be small enough to touch, and therefore activate gene expression, in a regionally restricted manner of the zebrafish embryo (approximately 30 μ m). The following materials were used to develop the wire probe: (2x) igniter wires (Fire Star 12" Igniter) which provides the heat, a J-type thermocouple (Omega 5TC-TT-J-36-36) for sensing the heat (Fig. A-2), a 50 AWG NiCr wire provided the heat at the tip, and a plastic holder.

One end of the igniter wire was soldered to the end of the 50AWG NiCr wire (Fig. A-3A) A small shrinking tube (3/64") was inserted to insulate the soldered part. A J-type thermocouple was then inserted right through the 50AWG NiCr wire. We used an Ohm-meter (Radio Shack) and measured about 2 Ohms on the 50 AWG NiCr wire and soldered it to the other igniter wire. The 50AWG NiCr wire was bent under the microscope using forceps in a "V" like shape (Fig.A-3B) and thermal epoxy adhesive (OMEGABOND® 101Two Part Epoxy) was applied to the heating probe body (covering the thermocouple's tip)(Fig.A-3C), but not to the naked NiCr tip. Figure A-3D shows a view of the finished heating probe that has an extremely small tip which easily heats up. In order for us to get the right shape of the microheater we needed to leave the applied epoxy to solidify for 4-6 hours.

The heating probe was secured to a plastic holder and attached to Narishigi

Micromanipulator. Before local heat shocking the embryos, we tested the temperature of the heating probe's tip by positioning the heating probe such that the very NiCr tip touched the K-type thermocouple's tip (which included a tiny drop of ERM). We then positioned the heating probe to the desired area on the embryo and applied heat. Figure A-4 shows a detailed schematic of the circuits that was used to build the control box.

GFP Antibody Labeling and Imaging

Following laser local heat shock, embryonic and larval staged zebrafish were fixed for 1 hour at room temperature in 4% paraformaldehyde and stored in methanol at -20°C. Zebrafish were incubated with anti-GFP (1:400, Molecular Probes) overnight at 4°C and detected using anti-rabbit IgG1 Alexa Fluor-488 (1:1000, Molecular Probes). Samples were cleared in 75% Glycerol and mounted in a petri dish (3cm) with no cover slip on top. For Z-stack images, larvae were mounted in 75% glycerol between two coverslips. 1 µm parasagittal sections were taken to include the entire depth of the GFP positive tissue using a Zeiss (Axioplan 2 Imaging) compound microscope, AxioVision 4.5 SPI. Images were color balanced using Adobe Photoshop CS2.

Results/Discussion

In order to test our heating devices on transgenic lines, we first global heat shocked the embryos. We needed to use control embryos that is to have embryos that are GFP positive (Fig. A-5A-C). The embryos that expressed GFP were selected and then incubated overnight so that they could be used the next day for the local heat shock. This provided a positive control for the proceeding local heat shock, by first ensuring that the

embryos carried the transgene. Embryos that were not green as seen in Figure A-5D (top embryo) were selected and separated from those that were GFP positive. Having global activation throughout the entire embryo and being able to look at the global activation of genes in the hind brain and otic placode as shown in Figures A-5E-F tells us that they surely could be used for the local heat shock application. These images show that the *hsp70* promoter is indeed activated allowing us to move on the next step where we tested for optimal local heat shock conditions using new tools and methods as described below.

We developed several wire heating probes and established optimal heat shock conditions for the heating probe that was the most stable. The criteria for a “stable heating probe” were a probe that consists of a material that has the right resistance properties, the size (AWG) of the actual tip was small and positioned closely to the thermocouple, and be able to activate the *hsp70* promoter without causing any cell death. The optimum temperature for wire heat shock was between the setting points of 47 °C - 49 °C (Table A-1).

It was necessary for us to tune up the control box before applying heat with the wire probe to the embryos. We therefore adjusted the setting point on the control box at a desired temperature and let the digital device auto tune for 5 to 10 minutes. We then used the Omega Digital Thermometer and tested the temperature at the tip of the heating probe. This test was done in one or two drops of agarose. The temperature of the setting point on the control box never matched the temperature of the tip of the microheater. The temperature of the NiCr wire probe always fluctuated and many times was below the setting point. We therefore had to increase the temperature of the setting point until we found the proper temperature of the heating probe’s tip.

The embryos that were heat shocked with the wire heating probe had the same range of age as the embryos used in the laser heat shock (48hpf to 148hpf). I initially performed pilot studies to ensure that the tip of the heating probe was not toxic to the embryo that touching the embryo with the probe does not induce stress, which may indirectly lead to the activation of gene expression. We first used control embryos that were transgenic GFP positive under the control of heat shock promoter *hsp70*, and left the wire-heating probe touching an area of the embryo with the heat turned off. The control embryos did not turn green, suggesting that physical manipulation of the embryo is not sufficient to illicit activation of the transgene, and the cells showed normal morphology. Every heating probe that was developed had its own setting point, since every NiCr wire that was soldered had a different resistance. Accordingly, because every tip was different, calibration was required each time a new tip was used. As shown in Figure A-6A, the activation of genes in the trunk was done by using one type of heating probe, while activation in the midbrain was performed using a different heating probe with a slightly different resistance, but same temperature at the tip (Figure A-6B). Some of the heating probes that were thicker in size (higher gauge) activated even a larger area (Figure A-6E), while others a smaller area (Figure A-6D&F).

Table A-1. Wire Local Heat Shock

Wire Type	Contr Box Temp.	Time (min) LHS	LHS Area	Activation Using Yes/No	Size of Area Activated Wire heat shock Dead Cells?
40AWG NiCr	50C	8'	HindBr.	Nothing	N/A
40AWG NiCr	55C	6'	Right Eye	Nothing	N/A
40AWG NiCr	55C	8'	HindBr.	Nothing	N/A
40AWG NiCr	49C	10'	Midbrain	Yes,	1/4 of midbrain about 30 μ m
40AWG NiCr	52C	7'	Heart	Yes	about 50 μ m
40AWG NiCr	49C	10'	Forebrain	Yes, but fluorecence dim	Yes, but fluorecence dim
40AWG NiCr	49C	10'	Midbrain	Nothing	N/A
40AWG NiCr	49C	NA	Midbrain	Yes	Yes, more towards the right eye
40AWG NiCr	49C	15'	Forebrain	Nothing	N/A
40AWG NiCr	50C	10	Hindbrain	Nothing	N/A
40AWG NiCr	49C	10'	Midbrain	Yes, 1/4 of midbrain is green	Yes, 1/4 of midbrain is green
40AWG NiCr	49C	15'	Hindbrain	nothing	N/A
40AWG NiCr	49C	15'	Midbrain	nothing	N/A
40AWG NiCr	49C	10'	Midbrain	yes, a very tiny spot	yes, a very tiny spot
40AWG NiCr	47C	10'	Midbrain	little bit, not very bright	little bit, not very bright
40AWG NiCr	47C	15'	Trunk	burned	burned
40AWG NiCr	47C	15'	Midbrain	nothing	N/A
40AWG NiCr	47C	10'	Trunk	nothing	N/A
40AWG NiCr	48C	15'	Trunk	nothing	N/A
40AWG NiCr	47C	10'	Midbrain	nothing	N/A
40AWG NiCr	48C	10'	Trunk	Yes, very bright green	Yes, very bright green
40AWG NiCr	47C	10'	ForeBrain	nothhing	N/A
40AWG NiCr	48C	10'	Tail	nothing	N/A
40AWG NiCr	48C	10'	Midbrain	nothing	N/A
40AWG NiCr	49C	10'	Midbrain	nothing	N/A
40AWG NiCr	46C	15'	Tale -tip	nothing	N/A
40AWG NiCr	48C	15'	Trunk-a	Yes	faint expression 70 μ m
40AWG NiCr	46C	15'	Trunk-c	nothing	N/A
40AWG NiCr	47C	15'	Midbrain	faint expression	faint expression
40AWG NiCr	49C	15'	trunk -b	nothing	N/A

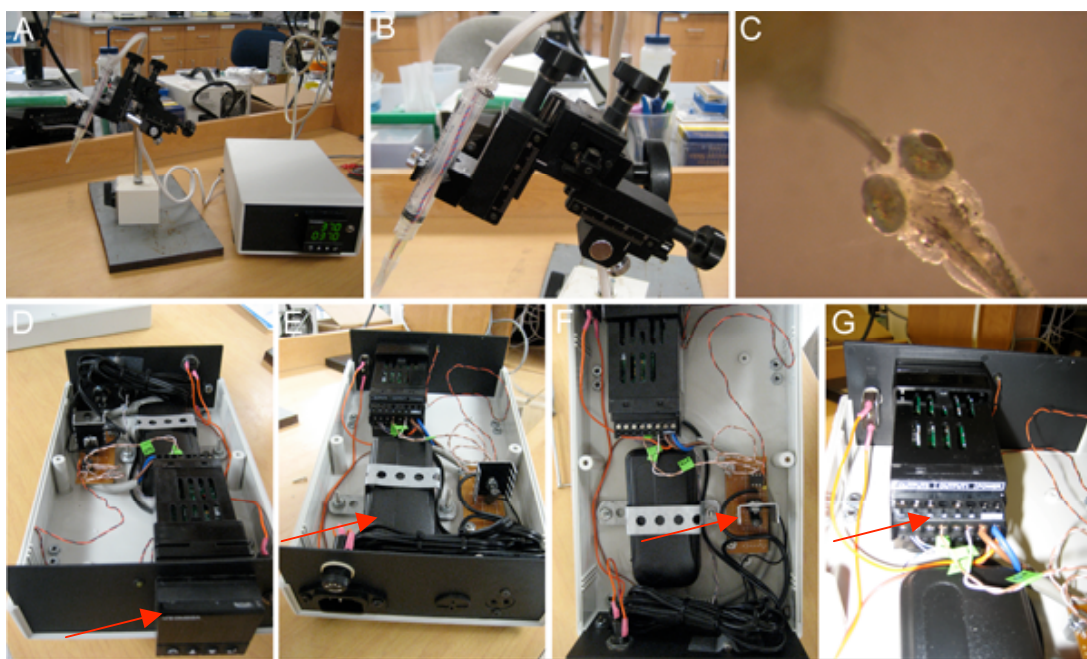


Figure A-1. Setup for the Wire Heat Shock Device.

(A) Shows the digital control box connected with wires to a microheater fixed on a micromanipulator. The setting point on the digital controller was manually adjusted. (B) A closer look at the wire heating probe that is held in a syringe. The micromanipulator was used for adjustment. (C) Shows wire heat shock on the forebrain on an anesthetized transgenic zebrafish larva (5 days post fertilization) mounted in agarose. (D) Is a frontal view of the control-box. The red arrow points at the Omega Digital Controller which controls the heat. (E) Dorsal view of the control-box. The red arrow is pointing at the 5V DC power supply held tight by a metal clamp. (F) A closer view the 1.8A solid-state relay. (G) Dorsal view of the digital controller. In the back of this digital device are inputs that connect to thermocouples and outputs that connect to igniter wires.

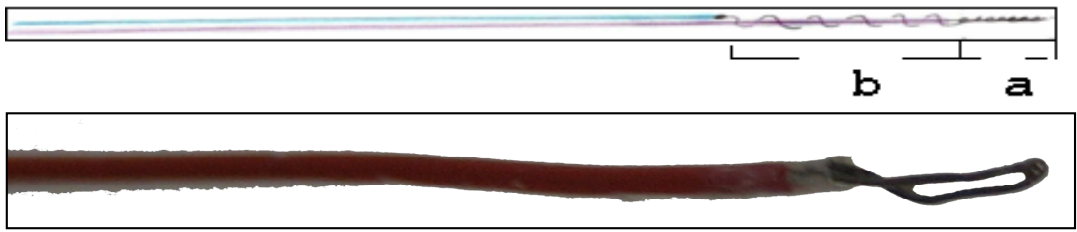


Figure A-2. Wires used to develop the heating probe.

The wire on top (red-blue color) is an igniter wire. The tip of the igniter wire is made of particle “a” and “b”. Particle “a” is a 40 AWG NiCr wire and particle “b” is the soldered part. Only particle “a” gives heat. The bottom wire (in red color) is a k-type thermocouple that was used to sense the heat. Only the very tip of the thermocouple senses the heat.

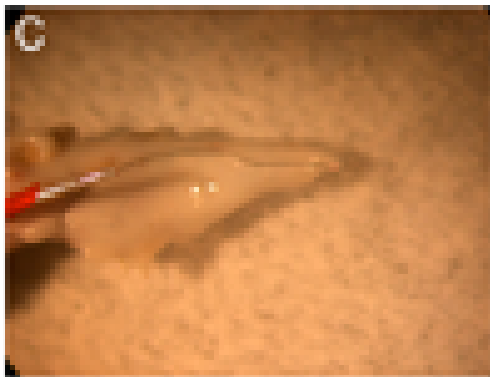
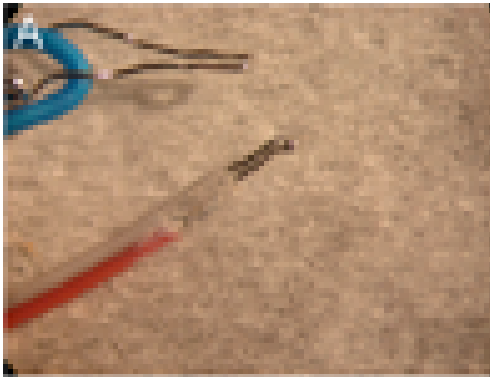


Figure A-3. Custom Fabrication of the wire heating probe.

(A) The tip of the igniter wire that consists of NiCr wire is coiled in a U-shape, similar to the thermocouple. (B) A 50 gauge NiCr wire is inserted into the thermocouple using surgical forceps. (C) Epoxy is applied to cover most of the heating probe. (D) The NiCr tip of the heating probe is less than 1 mm in length

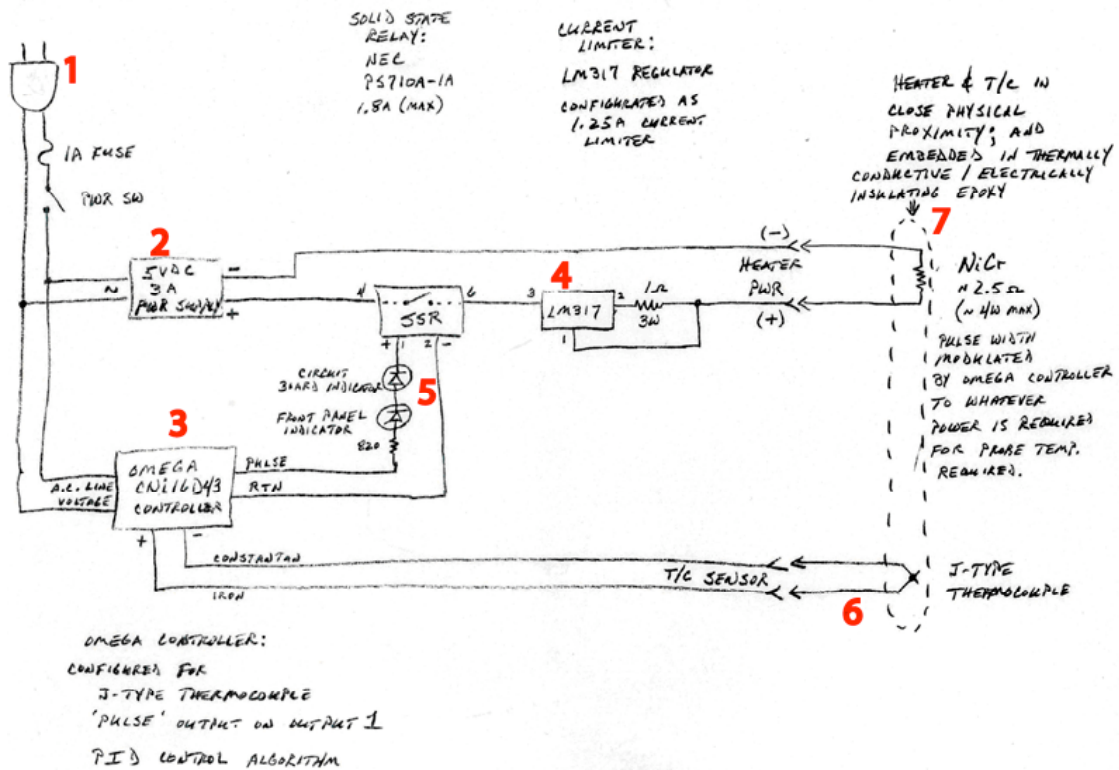


Figure A-4. Schematic of the entire wiring-circuit.

This figure a simplified hand-drawn schematic with all the essential electrical circuits and connectivity found inside the control box. (1) shows output of the AC. (2) is the DC adapter connected to the omega digital control device (3) in the power input and to the solid state relay (4). The Omega Digital Control Device has inputs for thermocouples and outputs for the igniter wires. (4) is the solid state relay to which the output wire is connected to transmit electrical impulses through igniter wires. (5) is a heat indicator that blinks every time heat is given off. (6) is the J-type thermocouple which will be adhered with epoxy to the end of the igniter wire. (7) is the end of the igniter wire that has a 50AWG NiCr wire that instantly heats up when electrical impulses come from the igniter wires (schematic supplied by Mike Conboy).

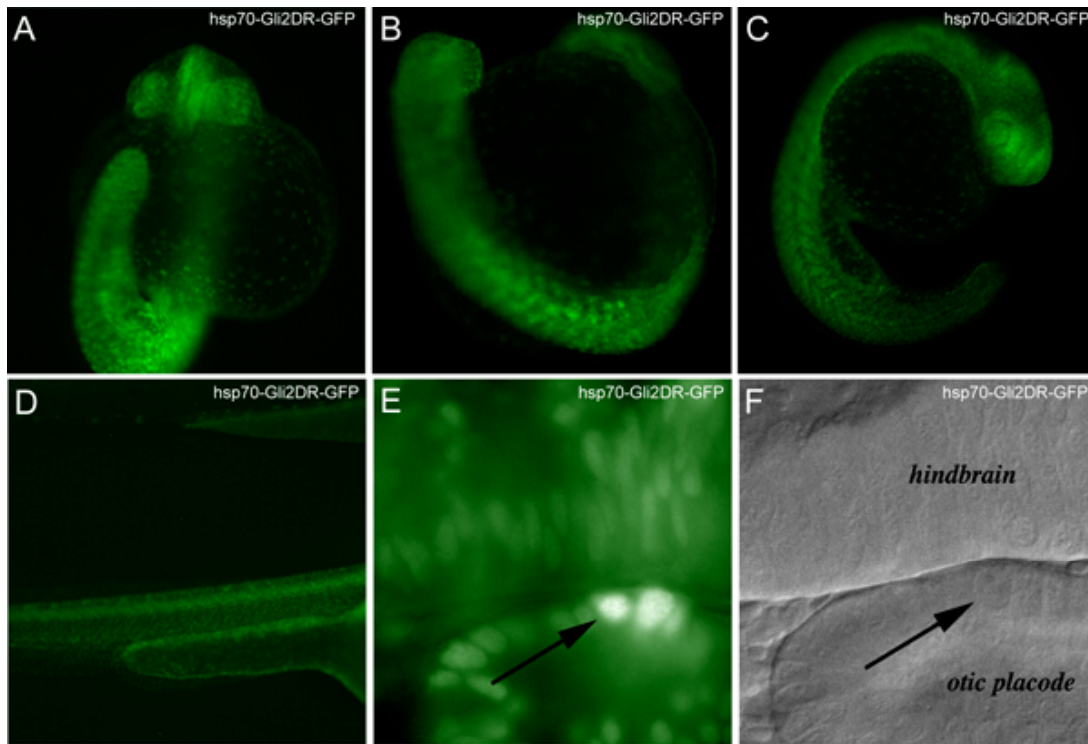


Figure A-5. Global Heat Shock.

(A) Fluorescent image of hsp70-Gli2DR-GFP transgenic zebrafish embryo at 18hpf, 4 hours after global heat shock (B) Fluorescent image of global heat shocked embryo at 18hpf. (C) Shows a clear image of embryo's somites visible in the trunk. (D) Shows two transgenic embryos that are 32hpf. The one on top is the control and the one on the bottom expresses GFP. (E) Arrow is pointing at individual nuclei that could be easily observed. This shows that it is nuclear localized. (F) Shows DIC image of the hindbrain and otic placode, it is the same image shown in (E).

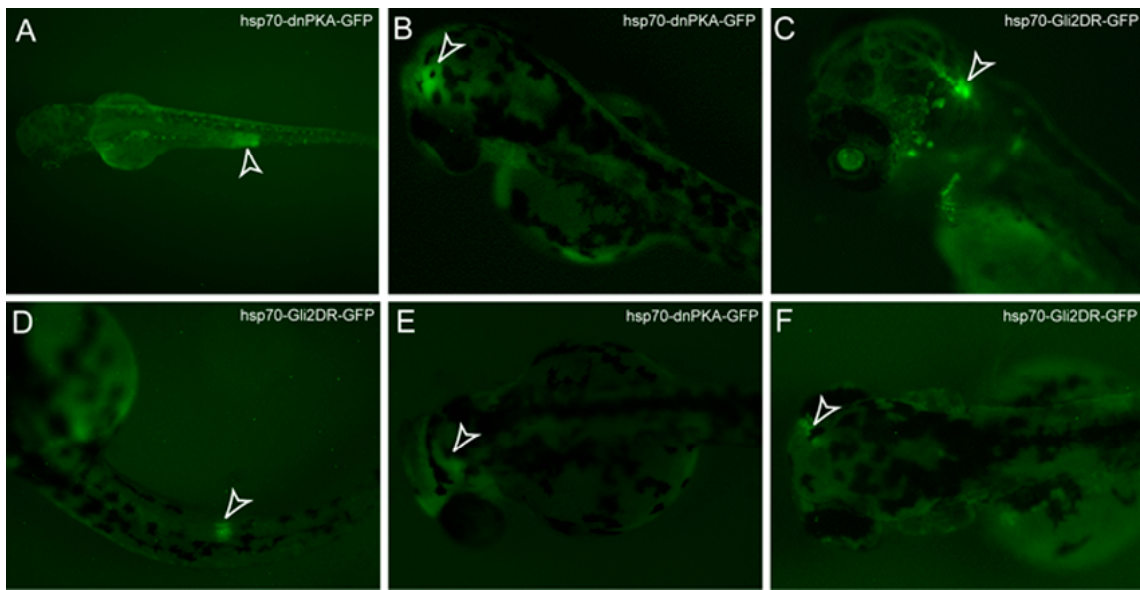


Figure A-6. Wire induction on six transgenic zebrafish.

(A) Embryo showing GFP expression in the trunk. The local heat shock time was 15 minutes with a setting point at 51 °C. (B) Dorsal view of a 72 hpf larva. The larva was heat shocked for 10 minutes having the setting point at 49 °C. GFP is detected in the midbrain. (C) Shows expression in the brain of a 72hpf larva. Total heat shock time was 15 minutes with a setting point at 49 °C. (D) The local heat shock time for this embryo was 20 minutes and the temperature on the setting point was 47 °C. (E) Shows GFP expression in larger area of the brain while (F) shows GFP expression in a smaller part of the brain.

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